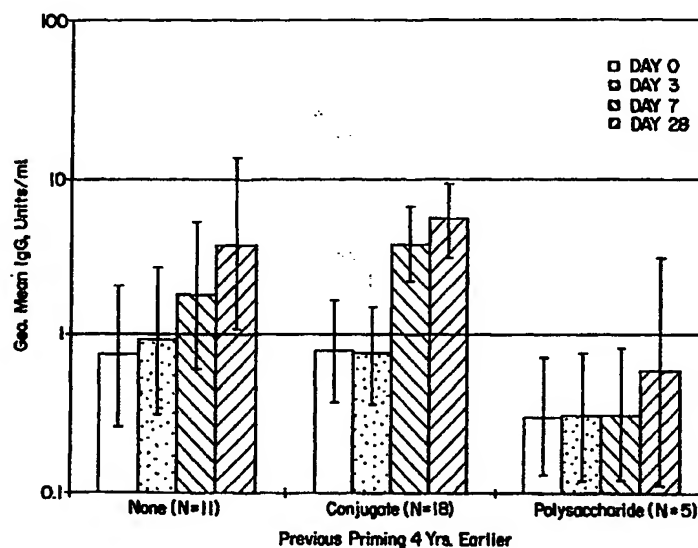




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(54) Title: METHODS OF IMMUNIZING ADULTS USING ANTI-MENINGOCOCCAL VACCINE COMPOSITIONS



## (57) Abstract

A method for boosting an immune response against meningococcal capsular antigen is disclosed. The method entails administering a first glycoconjugate vaccine composition to a subject to provide an initial state of anti-meningococcal immunity, and then boosting the anti-meningococcal immunity by administration of a second, boosting vaccination. Also disclosed is the use of vaccine compositions in the preparation of anti-meningococcal medicaments. The use entails administering a first glycoconjugate vaccine composition to a subject to provide an initial state of anti-meningococcal immunity, and then boosting the anti-meningococcal immunity by administration of a second, boosting vaccination.

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## 5 METHODS OF IMMUNIZING ADULTS USING ANTI-MENINGOCOCCAL VACCINE COMPOSITIONS

Cross-Reference to Related Application

10 This application is related to provisional  
patent application serial no. 60/050,581, filed June 24,  
1997, from which priority is claimed under 35 U.S.C.  
§119(e) (1) and which is incorporated herein by reference  
in its entirety.

15 Field of the Invention

The present invention relates to methods for  
immunizing a subject against meningococcal disease. More  
particularly, the invention relates to a method for  
avoiding immunological tolerance against meningococcal  
20 species in vaccinated subjects, using an anti-  
meningococcal glycoconjugate vaccine composition as the  
primary immunogen.

Background of the Invention

25 *Neisseria meningitidis* is an important cause of  
invasive bacterial disease, with an estimated 2600 cases  
of meningococcal meningitis occurring in the United  
States each year, primarily in children and young adults  
(Jafari et al. (1997) *MMWR* 46:1-10; Perkins et al. (1997)  
30 *MMWR* 46:13-21). In England and Wales, there has been a  
steady rise in reported cases of meningococcal disease  
since 1984, peaking at greater than 2000 cases per year  
in 1991 and 1992 (Jones, D. (1995) "Epidemiology of  
Meningococcal Disease in Europe and the USA, in  
35 *Meningococcal disease*, Cartwright, K. (ed), John Wiley &

Sons Ltd., pp. 147-157). Despite antimicrobial therapy, mortality rates in Europe and in the USA from meningococcal disease remain high (i.e., 12 to 14 percent of cases, Jafari, *supra* and Jones, *supra*.) In most  
5 industrialized countries, the vast majority of isolates causing meningococcal disease are serogroups B or C (Jones, *supra*; Harrison, L. (1995) *JAMA* 273:419-421; Jackson et al. (1995) *JAMA* 273:383-389; Whalen et al. (1995) *JAMA* 273:390-394). For reasons that are unknown,  
10 serogroup A strains, the major cause of disease in developing countries (Harrison, *supra*) are very rare in industrialized countries.

A substantial portion of meningococcal disease is potentially preventable by vaccination (Artenstein et al. (1970) *N. Engl. J. Med.* 282:417-420; Reingold et al. (1985) *Lancet* 2:114-118; Peltola et al. (1977) *N. Engl. J. Med.* 297:686-691). Effective polysaccharide vaccines against disease caused by serogroup A and C strains have been available for more than 20 years and, more recently,  
20 tetravalent vaccines have been licensed for prevention of serogroups A, C, Y and W135 isolates (Armand et al. (1982) *J. Biol. Stand.* 10:335-339; Ambrosch et al. (1983) *Bull. World Health Organ.* 61:317-323).

Despite their general availability,  
25 meningococcal polysaccharide vaccines are used infrequently in industrialized societies (Harrison, *supra*). For example, in the United States, vaccination is largely limited to certain high risk situations, such as with patients with functional asplenia or terminal  
30 complement deficiency diseases (Jafari, *supra*). Vaccination is also used for controlling meningococcal disease in military recruits (Harrison, *supra*), and may be beneficial for healthy individuals traveling to hyperendemic areas, and for control of civilian outbreaks  
35 of meningococcal disease caused by serogroup strains

included in the available vaccines (Perkins, *supra*;  
Masterton et al. (1988) *J. Infect.* 17:177-182). The  
reasons for the limited use of meningococcal  
polysaccharide vaccines in the general population include  
5 their poor immunogenicity in infants less than 2 years of  
age, the age group at greatest risk of developing  
meningococcal disease (Jafari, *supra*; Jones, *supra*). In  
addition, the duration of vaccine-induced protection  
elicited in older children and adults is limited  
10 (Zangwill et al. (1994) *J. Infect. Dis.* 169:847-852).  
Finally, these polysaccharide vaccines provide no  
protection against disease caused by serogroup B strains,  
which accounts for approximately 40% of all cases in the  
United States (Jafari, *supra*) and Canada (Whalen, *supra*),  
15 and an even greater proportion in the United Kingdom  
(Jones, *supra*).

More effective polysaccharide-protein conjugate  
vaccines for prevention of disease caused by  
meningococcal A and C strains are currently under  
20 development (reviewed in Granoff et al. (1997) *Int. J.*  
*Infect. Dis.* 1:152-157). These conjugate vaccines are  
immunogenic in infants and toddlers, and elicit high  
titers of serum bactericidal antibody (Fairley (1996) *J.*  
*Infect. Dis.* 174:1360-1363, and Lieberman et al. (1996)  
25 *JAMA* 275:1499-1503).

In addition, polysaccharide derivatives have  
been prepared to circumvent disease caused by  
meningococcal B strains. For example, C<sub>4</sub>-C<sub>8</sub> N-acyl-  
substituted polysaccharide derivatives have been  
30 described. See, EP Publication No. 504,202 B, to  
Jennings et al. Similarly, U.S. Patent No. 4,727,136 to  
Jennings et al. describes an N-propionylated  
meningococcal B polysaccharide molecule. Mice immunized  
with glycoconjugates formed with these polysaccharide  
35

derivatives were reported to elicit high titers of IgG antibodies. Jennings et al. (1986) *J. Immunol.* 137:1708. More recently, meningococcal B oligosaccharide derivative fragments, and glycoconjugates made from those fragments, have been shown to be highly effective immunogens for use in anti-meningococcal B vaccine preparations. International Publication No. WO 98/086543.

Although anti-meningococcal conjugate vaccine preparations are more effective than unconjugated vaccines in infants and toddlers, unconjugated polysaccharide vaccines are highly immunogenic in adults, eliciting effective short-term protection against disease (Artenstein, *supra.*, Gold et al. (1971) *Bull. World Health Organ.* 45:279-282). Thus, there appears to be no real advantage to using the more costly meningococcal conjugate vaccine in adults. Indeed, in comparative immunogenicity studies, the magnitude of the serum antibody response of adults given a dose of unconjugated pneumococcal or meningococcal polysaccharide vaccine appears to be similar to that elicited by the corresponding polysaccharide-protein conjugate vaccine (Anderson et al. (1994) *Infect. Immun.* 62:3391-3395; Powers et al. (1996) *J. Infect. Dis.* 173:1014-1018).

#### Summary of the Invention

It is a primary object of the invention to provide a method for boosting in an adult subject an immune response against meningococcal capsular antigen. The method generally entails the steps of (a) administering a first vaccine composition to an adult subject in order to elicit an immune response against a meningococcal species, and (b) administering a second vaccine composition to said adult subject in order to boost the anti-meningococcal response. The first vaccine composition comprises a meningococcal oligosaccharide

conjugated to a carrier molecule, wherein the composition is administered in an amount sufficient to elicit an anti-meningococcal immune response, and said immune response is boostable upon revaccination with a second meningococcal vaccine composition. The second vaccine composition is administered to the subject after serum anti-meningococcal antibody concentration induced by the first vaccine composition have declined to subprotective levels.

It is also an object of the invention to provide a use of a first and second meningococcal polysaccharide or oligosaccharide composition in the preparation of a medicament. The first composition comprises a meningococcal oligosaccharide conjugated to a carrier molecule, and is administered in an amount sufficient to elicit an anti-meningococcal immune response which is boostable upon re-vaccination with a second meningococcal vaccine composition. The second composition comprises a meningococcal polysaccharide or oligosaccharide immunogen, and is administered to the subject after serum anti-meningococcal antibody concentrations induced by the first vaccine composition have declined to sub-protective levels.

It is an advantage of the present invention that the methods and uses can be employed in an anti-meningococcal vaccination protocol which avoids problems associated with induction of immunological tolerance to meningococcal immunogens as seen with prior vaccination strategies.

It is also a feature of the present invention that a wide variety of commonly available anti-meningococcal capsular oligosaccharide or polysaccharide glycoconjugates may be used as the immunogen in the first vaccine composition.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned by practice of the invention.

#### Brief Description of the Figures

Figure 1 depicts the geometric mean antibody response to a booster dose of the quadravalent meningococcal A, C, Y, W135 polysaccharide vaccine (Menomune®) in naive subjects, and in subjects previously given either (a) a full dose of the tetravalent meningococcal polysaccharide vaccine (Menomune®), or (b) a dose of an investigational meningococcal A and C oligosaccharide-protein conjugate vaccine.

#### Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional methods of immunology, microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); *DNA Cloning: A Practical Approach*, vol. I & II (D. Glover, ed.); *Oligonucleotide Synthesis* (N. Gait, ed., 1984); *Nucleic Acid Hybridization* (B. Hames & S. Higgins, eds., 1985); *Transcription and Translation* (B. Hames & S. Higgins, eds., 1984); *Animal Cell Culture* (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).



All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

As used in this specification and the appended  
5 claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

#### I. Definitions

10 In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

An "antigen" is defined herein to include any substance that may be bound by an antibody molecule. An  
15 "immunogen" is an antigen that is capable of initiating lymphocyte activation resulting in an antigen-specific immune response. Such activation generally results in the development of a secretory, cellular and/or antibody-mediated immune response against the immunogen.  
20 Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies from any of the immunological classes, such as IgA, IgD, IgE, IgG or IgM; the proliferation of B and T lymphocytes; the provision of activation, growth and  
25 differentiation signals to immunological cells; expansion of helper T cell, suppressor T cell, and/or cytotoxic T cell and/or  $\gamma\delta$  T cell populations. Immunogens therefore include any molecule which contains one or more antigenic determinants (e.g., epitopes) that will stimulate a  
30 host's immune system to initiate such an antigen-specific response.

By "epitope" is meant a site on an antigen to which specific B cells and T cells respond. The term is also used interchangeably with "antigenic determinant" or  
35 "antigenic determinant site." A peptide epitope can

comprise 3 or more amino acids in a spatial conformation unique to the epitope. Generally, an epitope consists of at least 5 such amino acids and, more usually, consists of at least 8-10 such amino acids. Methods of  
5 determining spatial conformation of amino acids are known in the art and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. Furthermore, the identification of epitopes in a given protein is readily accomplished using  
10 techniques well known in the art. See, e.g., Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998 (general method of rapidly synthesizing peptides to determine the location of immunogenic epitopes in a given antigen); U.S. Patent No. 4,708,871 (procedures for identifying and  
15 chemically synthesizing epitopes of antigens); and Geysen et al. (1986) *Molecular Immunology* 23:709-715 (technique for identifying peptides with high affinity for a given antibody). Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the  
20 ability of one antibody to block the binding of another antibody to a target antigen.

As used herein, "treatment" refers to any of (i) prevention of infection or reinfection, as in a traditional vaccine, (ii) reduction or elimination of  
25 symptoms, and (iii) reduction or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

By "mammalian subject" is meant any member of  
30 the class *Mammalia*, including, without limitation, humans and other primates, including such non-human primates as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; and laboratory  
35 animals including rodents such as mice, rats and guinea

pigs. The term does not denote a particular age or sex. Thus, both adult and newborn individuals, as well as fetuses, either male or female, are intended to be covered.

5

## II. Modes of Carrying Out the Invention

The present invention is premised, in part, on the unexpected discovery that use of an anti-meningococcal conjugate vaccine composition in adults  
10 (instead of an unconjugated anti-meningococcal polysaccharide vaccine) induces polysaccharide-responsive memory B cells and long-term immunologic memory in vaccinated subjects, both of which factors contribute to more robust and durable protection against meningococcal  
15 disease. In fact, it has surprisingly been found that the anti-meningococcal immune response in adults vaccinated with a conjugate vaccine formulation is readily boostable upon re-vaccination with a second anti-meningococcal vaccine composition.

20 In contrast, it has also been found herein that vaccination with an unconjugated tetravalent meningococcal A, C, Y, W135 polysaccharide vaccine (Menomune®, Connaught Laboratories, Inc., Swiftwater PA) induces immunologic paralysis of toddlers and adults to  
25 meningococcal polysaccharides. More particularly, meningococcal C vaccination with a polysaccharide vaccine (unconjugated) in subjects during the first six months of age results in depression of serum antibody responses to a booster vaccination with meningococcal C polysaccharide  
30 given 6 months later (when compared to the responses of infants of similar age vaccinated for the first time). In the study described hereinbelow, infants were given two doses of a meningococcal A and C polysaccharide vaccine at 3 and 6 months of age and boosted with a third  
35 injection at 18 to 24 months of age. As shown in Figure

1, the geometric mean antibody response to the booster dose was nearly 10-fold lower than that of control children of the same age vaccinated for the first time. This new information on induction of immunologic tolerance in these vaccinated subjects indicates that such tolerance is not limited to infants less than 6 months of age, but also occurs in toddlers vaccinated at 15 to 23 months, and in adults. In fact, antibody refractoriness in adult subjects was observed 4 years after a polysaccharide vaccination.

The induction of immunologic tolerance to meningococcal species in previously vaccinated subjects is of significant clinical importance. In this regard, data from experimental animals indicate that mice tolerized to pneumococcal polysaccharide show increased lethality from experimental challenge with pneumococci possessing the homologous serotype (reviewed in Halliday, W. (1971) *Bacteriol. Rev.* 35:267-289). This increased susceptibility may be a result of an impaired ability to mount serum anticapsular antibody responses upon exposure to the encapsulated bacteria. In humans, the contemporary knowledge accepts that unconjugated meningococcal polysaccharide vaccine are protective in adults and, possibly, in older children. However, this knowledge is generally based upon efficacy data in adults that were obtained from studies performed in military recruits in which follow-up was very short (8 weeks). As has been discovered herein, there may be a late-onset increased risk of disease in vaccinated subjects as a result of immune refractoriness to meningococcal polysaccharides, once increased serum antibody concentrations induced by vaccination have declined to sub-protective levels (i.e., after about 3 years). The impaired meningococcal C serum bactericidal antibody responses of toddlers and adults previously vaccinated

with the tetravalent polysaccharide vaccine is consistent with this possibility.

Taken together, the data presented herein raise a safety concern for the use of unconjugated anti-meningococcal vaccines, as this product is recommended in the United States for use in children two years of age or older, but the vaccine also can be used in infants and younger children to control outbreaks of disease (Jafari, *supra* and Perkins, *supra*).

Accordingly, it is a primary object of the invention to provide a method for boosting in an adult subject an anti-meningococcal immune response against a meningococcal capsular polysaccharide antigen. The method generally entails a primary vaccination using a anti-meningococcal glycoconjugate vaccine composition which comprises meningococcal capsular polysaccharide antigen derived from one or more meningococcal species (i.e., a monovalent or polyvalent vaccine composition) conjugated to an appropriate carrier molecule. The primary vaccination is sufficient to elicit an anti-meningococcal immune response in the vaccinated subject which is specific for one or more meningococcal species. After the immune response elicited by the primary vaccination has declined to subprotective levels, a boosting vaccination is performed in order to provide a boosted anti-meningococcal immune response.

The anti-meningococcal glycoconjugates used for the primary vaccination are prepared using carrier molecules that will not themselves induce the production of harmful antibodies. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or liposomes), and inactive virus particles. Preferably, capsular

meningococcal polysaccharide or oligosaccharide molecules containing at least one immunologically relevant epitope are conjugated to a bacterial toxoid carrier molecule, such as, but not limited to, a toxoid from diphtheria, 5 tetanus, cholera, etc. In particular embodiments, capsular polysaccharide molecules are coupled to the CRM<sub>197</sub> protein carrier. The CRM<sub>197</sub> carrier is a well-characterized non-toxic diphtheria toxin mutant that is useful in glycoconjugate vaccine preparations intended 10 for human use. (Bixler et al. (1989) *Adv. Exp. Med. Biol.* 251:175, and Constantino et al. (1992) *Vaccine*). In other embodiments, glycoconjugates are formed with protein carriers known to have potent T-cell epitopes. Exemplary carriers include, but are not limited to, 15 Fragment C of tetanus toxin (TT), and the Class 1 or Class 2/3 OMPs of *N. meningitidis*. Such carriers are well known to those of ordinary skill in the art.

In particular embodiments of the invention, the primary vaccination entails administration of a 20 meningococcal A and C oligosaccharide-based glycoconjugate vaccine composition as described by Anderson et al. (*supra*). In other related embodiments, the primary vaccination is given using a meningococcal B oligosaccharide-based glycoconjugate as described in 25 International Publication No. WO 98/086543, which publication is incorporated herein by reference in its entirety. Other vaccine compositions that can be used herein for the primary vaccination include, for example, glycoconjugates based on meningococcal B polysaccharide 30 derivatives (e.g., those described in EP Publication No. 504,202 B and U.S. Patent No. 4,727,136, both of which are incorporated herein by reference), and monovalent meningococcal C or trivalent meningococcal A, B and C oligosaccharide-based glycoconjugates.

The secondary (boosting) vaccination can be carried out using any suitable anti-meningococcal vaccine composition; however, the second vaccine composition is preferably also a meningococcal capsular polysaccharide-  
5 or oligosaccharide-based conjugate in order to avoid the possibility of immunologic tolerance associated with unconjugated anti-meningococcal vaccine compositions.

As will be known by those skilled in the art upon reading the instant specification, several factors  
10 will have an impact on the physical and immunological properties of the above-described glycoconjugates. Specifically, the ratio of oligosaccharide (and/or polysaccharide)-to-protein (hapten loading density), linkage chemistry, and the choice of carrier moiety are  
15 all factors that should be considered and optimized in the preparation of the glycoconjugates used in the methods herein. For example, a low saccharide loading density may result in poor anti-saccharide antibody response. On the other hand, a heavy loading of  
20 saccharides could potentially mask important T-cell epitopes of the protein molecule, thus abrogating the carrier effect and attenuating the total anti-saccharide immune response.

Accordingly, during glycoconjugate production,  
25 aliquots can be withdrawn and analyzed by SEC-HPLC in order to monitor the extent of the conjugation process. The use of a disaggregating buffer, for example EDTA, SDS, deoxycholate, or the like, can be employed to separate components possibly adhering to the preparations  
30 by non-covalent interactions. To ensure glycosylation of the carrier, the shift in retention time of the particular protein carrier toward the exclusion volume ( $V_0$ ) of the column can be monitored. In addition, a gradual reduction of the saccharide peak area in a HPLC

chromatogram can be used to indicate incorporation of the saccharide onto the carrier.

Post-production characterization of the glycoconjugates can include molecular weight determination using, for example, gel filtration columns. Further characterization may also include electrophoretic mobility on SDS-PAGE separation equipment and analysis of chemical composition of the glycoconjugates with respect to carbohydrate and amino acid components. The identity of product purity, and the absence of residual contaminants (such as nucleic acids, LPS, and free saccharides and/or carrier) can also be verified using known techniques. Confirmation of stable covalent attachment can be accomplished using a combination of analytical techniques, including gel filtration in detergent-containing buffer, SDS-PAGE followed by Western Blot analysis and amino acid analysis. See, e.g., Vella et al. (1992) *Vaccines: New Approaches to Immunological Problems*, (Ellis, R.W. ed), Butterworth-Heinemann, Boston, pp 1-22, Seid et al. (1989) *Glycoconjugate J.* 6:489.

The anti-meningococcal vaccine compositions used in the primary and subsequent (boosting) vaccinations can further be administered in conjunction with other antigens and immunoregulatory agents, for example, immunoglobulins, cytokines, lymphokines, and chemokines, including but not limited to IL-2, modified IL-2 (cys125→ser125), GM-CSF, IL-12,  $\gamma$ -interferon, IP-10, MIP1 $\beta$  and RANTES.

The vaccine compositions will generally include one or more "pharmaceutically acceptable excipients or vehicles" such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.



Adjuvants may also be used to enhance the effectiveness of the vaccines. Adjuvants can be added directly to the vaccine compositions or can be administered separately, either concurrently with or shortly after, administration of the vaccines. Such adjuvants include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (International Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) Ribit<sup>™</sup> adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox<sup>™</sup>); (3) saponin adjuvants, such as Stimulon<sup>™</sup> (Cambridge Bioscience, Worcester, MA) may be used or particle generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc.; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera

toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63 (where lysine is substituted for the wild-type amino acid at position 63) LT-R72 (where arginine is substituted for the wild-type amino acid at position 72), CT-S109 (where serine is substituted for the wild-type amino acid at position 109), and PT-K9/G129 (where lysine is substituted for the wild-type amino acid at position 9 and glycine substituted at position 129) (see, e.g., International Publication Nos. W093/13202 and W092/19265); and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition.

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

Typically, the vaccine compositions are prepared as injectables, either as liquid solutions or suspensions; or as solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect.

The vaccine compositions will comprise a therapeutically effective amount of one or more meningococcal capsular oligosaccharide or polysaccharide immunogens, and any other of the above-mentioned components, as needed. By "therapeutically effective amount" is meant an amount of a molecule which will induce an immunological response in the individual to which it is administered without stimulating an autoimmune response. Such a response will generally result in the development in the subject of a secretory, cellular and/or antibody-mediated immune response to the

vaccine. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies from any of the immunological classes, such as immunoglobulins A, D, E, G or M; the  
5 proliferation of B and T lymphocytes; the provision of activation, growth and differentiation signals to immunological cells; expansion of helper T cell, suppressor T cell, and/or cytotoxic T cell and/or  $\gamma\delta$  T cell populations.

10 Preferably, the effective amount is sufficient to bring about treatment, i.e., reduction or complete elimination of symptoms, or prevention of disease symptoms. The exact amount necessary will vary depending on the subject being treated; the age and general  
15 condition of the subject to be treated; the capacity of the subject's immune system to synthesize antibodies; the degree of protection desired; the severity of the condition being treated; the particular molecule selected and its mode of administration, among other factors. An  
20 appropriate effective amount can be readily determined by one of skill in the art. A "therapeutically effective amount" will fall in a relatively broad range that can be determined through routine trials. More particularly, the meningococcal capsular oligosaccharide or  
25 polysaccharide immunogens will be administered in a therapeutically effective amount that comprises from about 0.1  $\mu\text{g}$  to about 100 mg, more preferably from about 0.5  $\mu\text{g}$  to about 1 mg, and most preferably about 1  $\mu\text{g}$  to about 500  $\mu\text{g}$  of the oligosaccharide or polysaccharide  
30 immunogen delivered per dose.

Once formulated, the vaccine compositions are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Alternative formulations suitable for other modes of  
35 administration include oral and pulmonary formulations,

suppositories, and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule.

5    III. Experimental

          The following studies were designed to assess whether meningococcal conjugate vaccination of adults induces immunologic memory to unconjugated meningococcal C polysaccharide. To address this question, adults who  
10    had been immunized three to four years earlier with an investigational meningococcal A and C conjugate vaccine were re-vaccinated with unconjugated tetravalent meningococcal polysaccharide vaccine. The serum antibody responses to this booster injection were compared to  
15    those of previously unvaccinated adults, or those of adults previously vaccinated with unconjugated meningococcal polysaccharide vaccine. Since "unprimed" adults were expected to show very high serum antibody responses to unconjugated meningococcal polysaccharide  
20    vaccine, the dose of meningococcal polysaccharide vaccine that was used for the booster injection was chosen such that it would normally be considered suboptimally immunogenic (1/50 of the usual dose, to serve as a probe of B cell immunologic memory).

25

A. Methods

Subjects: The following study was approved by the Saint Louis University Institutional Review Board. Thirty-four healthy adults, ages 20 to 53 years, were  
30    divided into three groups based on their previous meningococcal vaccination histories. Group 1 consisted of 5 subjects, each of whom had received a full dose of a U.S. licensed tetravalent meningococcal polysaccharide vaccine (Menomune®, 50 µg of A, C, Y, and W135  
35    polysaccharides per 0.5 ml dose). This dose was given

four years earlier as part of a previous study (Anderson, *supra*). Group 2 consisted of 18 subjects who had received a dose of an investigational meningococcal A and C oligosaccharide-protein conjugate. Fifteen of these  
5 subjects had been immunized four years earlier as part of the same study (Anderson, *supra*). The remaining three subjects were vaccinated three years earlier in a subsequent trial (Anderson et al., unpublished data).

The conjugate vaccine used in the studies  
10 contained 22  $\mu$ g each of Group A and C oligosaccharides and 48.7  $\mu$ g of CRM<sub>197</sub> protein (a cross-reactive mutant nontoxic diphtheria toxin). Four of the subjects in group 2 received the full 22  $\mu$ g dose, 11 received an 11  $\mu$ g dose (including the three subjects vaccinated in the  
15 second trial), and 3 received a 5.5  $\mu$ g dose. All doses were adsorbed to 1 mg of aluminum hydroxide and given in a 1 ml dose. In the present study, the magnitude and kinetics of the booster antibody responses of the subjects previously given different doses of conjugate  
20 vaccine were very similar. Therefore, for presentation of the results, the data from the three priming doses were combined. Group 3 consisted of 11 adults who had not previously received meningococcal vaccine. Two of the subjects in this group had been randomized in the  
25 first study to receive a saline placebo injection (Anderson, *supra*), and the remaining 9 subjects were previously unvaccinated healthy adults recruited as controls for the present booster study.

The demographic characteristics of the three  
30 "priming" vaccine groups were similar with respect to median age at the time of the booster vaccination (38, 36, and 40 years of age for groups 1, 2, and 3, respectively), gender (predominantly female: 100%, 89%, and 82%, respectively), and race distribution (white:  
35 100%, 94%, and 91%, respectively).

Vaccinations: After informed consent, all 34 healthy adults in groups 1, 2 and 3 were vaccinated with 1/50 of the usual dose of a quadravalent meningococcal A, C, Y, and W135 polysaccharide vaccine (1 ml containing 1  
5  $\mu$ g of each polysaccharide, given IM in the deltoid). To prepare this dose, lyophilized meningococcal polysaccharide vaccine (Menomune®) from Connaught Laboratories (Swiftwater, PA, U.S.) was reconstituted with 0.6 ml of diluent provided by the manufacturer. The  
10 resulting solution contained 100  $\mu$ g/ml of each of the polysaccharides. From this solution, 0.5 ml was diluted into 49.5 ml of preservative-free saline for injection, to yield the 1 ml dose. Serum samples were obtained immediately prior to vaccination (time 0), and 3, 7 and  
15 28 days later, for measurement of antibody response to the meningococcal C component of the vaccine.

Immunoassays: All assays were performed "blindly" on coded serum samples. Serum anti-*N meningitidis* group C polysaccharide antibody  
20 concentrations were measured by an enzyme-linked immunosorbent assay (ELISA), adapted from a method previously described (Granoff et al. (1997) *Infect. Immun.* 65:1710-1715). In the present study, the assay employed an alkaline-phosphatase conjugated mouse  
25 monoclonal antibody specific for human IgG (clone HP6083) (Granoff et al. (1995) *Clin. Diagn. Lab. Immunol.* 1:574-582). Also, the buffer for diluting the serum samples contained 75 mM sodium thiocyanate, which favored the detection of high avidity anticapsular antibodies as  
30 compared to low avidity antibodies (Raff et al. (1996) "Correlation between ELISA and bactericidal activity in infants and toddlers immunized with a MenC-CRM conjugate vaccine," in *Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy*, page  
35 158 (Abstract)). The IgG meningococcal C anticapsular

antibody concentrations in test samples are reported in arbitrary units per ml, compared to that present in an internal reference serum pool prepared from serum samples from vaccinated healthy adults. For comparison, the  
5 meningococcal reference serum pool CDC1992 (provided by George Carlone, Centers for Disease Control and Prevention, Atlanta, Georgia) (Gheesling et al. (1994) *J. Clin. Microbiol.* 32:1475-1482) contained 19.1 units/ml of the meningococcal C anticapsular antibody as measured by  
10 this modified assay.

Complement-mediated bactericidal antibody to *Neisseria meningitidis* group C was assayed as previously described, (Granoff et al. (1997) *Infect. Immun.* 65:1710-1715; and Mandrell et al. (1995) *J. Infect. Dis.*  
15 172:1279-1289) with the following modifications. The group C test organism (*N. meningitidis* group C, strain 60E, obtained from Dr. W. Zollinger, Walter Reed Institute for Medical Research, Washington, DC) was grown for approximately 2 hours in Muller Hinton (MH) broth  
20 containing 0.25% glucose, which rendered the organism resistant to complement-mediated bacteriolysis by endogenous "natural" antibodies, as compared to organisms grown in Muller Hinton without supplemental glucose. All test sera were heated at 56°C for 30 mins to  
25 inactivate endogenous complement. The complement source for the bactericidal assay was pooled sera obtained from three healthy adults who had no detectable anticapsular antibody to meningococcal C, and whose sera lacked intrinsic bactericidal activity when tested at 40  
30 percent. In the bactericidal assay, this complement source was used at 20 percent in the final reaction mixture, along with serial 2-fold dilutions of test sera beginning at a 1:8 dilution (12.5 percent in the final reaction) and Gey's buffer (instead of barbitol buffer as  
35 previously described) (Mandrell, *supra*). Serum

bactericidal titers were defined as the dilution of test sera resulting in a 50% decrease in colony forming units per ml after 60 minutes incubation of bacteria in the reaction mixture, compared to control bacteria at time 0.

5 Note that the titers reported with this assay tend to be lower than those described in previous studies (Anderson, *supra* and Maslanka et al. (1997) *Clin. Diagn. Lab. Immunol.* 4:156-167). The principal reasons are: (1) the use of a test organism grown with supplemental glucose;

10 (2) the use of Gey's buffer instead of barbital buffer (Mandrell, *supra*) and (3) the use of human as opposed to rabbit complement, since in previous studies rabbit complement was shown to amplify greatly bactericidal activity of human antibodies (Mandrell, *supra* and

15 Zollinger et al. (1983) *Infect. Immun.* 40:257-264). Human complement also was chosen for the present study because the data demonstrating that serum bactericidal antibody correlated with protection of humans against invasive meningococcal disease were derived from studies

20 that used human complement (Goldschneider et al. (1969) *J. Exp. Med.* 129:1307-1326).

Statistical analysis: Antibody concentrations were transformed ( $\log_{10}$ ). For these calculations, bactericidal titers less than 1:8 were assigned as 1:4,

25 and IgG antibody concentrations less than 0.4 units/ml were assigned a value of 0.2 units/ml. Geometric means and 95% confidence intervals were computed by using the log transformed means and standard errors were computed from a one-way analysis of variance (ANOVA) model.

30 Differences between each pair of groups with respect to geometric means were tested by using the P values from the ANOVA model.

#### B. Results



Clinical tolerability: Vaccination with 1/50 of the usual dose of Menomune was well tolerated irrespective of previous meningococcal vaccination status. During the 28 days of follow-up, there were no clinically significant local reactions at the injection site or systemic reactions, such as fever, rash, or myalgia, in any of the 34 subjects.

Antibody response: Figure 1 shows the geometric mean IgG anticapsular antibody responses of each group to the booster vaccination. Prior to the vaccination, there were no significant differences between the geometric mean antibody concentrations of the three groups (0.30, 0.78 and 0.73 units/ml, for groups 1, 2, and 3, respectively). At 3 days after vaccination, there was no evidence of a significant increase in serum IgG antibody concentrations in any of the groups, when compared to the respective antibody concentrations present in pre-vaccination sera. However, by 7 days, subjects in group 2, who previously had received the conjugate vaccine, and subjects in group 3, who were vaccinated for the first time, showed significant IgG responses, compared to their respective IgG serum antibody concentrations present at time 0 ( $p < 0.05$  for each group). In contrast, the adults in group 1, who had received a full dose of licensed meningococcal A and C polysaccharide vaccine 4 years earlier, showed no evidence of an IgG response at 7 days (geometric mean IgG antibody concentration of 0.38 units/ml at 7 days vs. 0.42 units/ml at time 0). Similarly, there was no significant increase in geometric mean antibody concentration in this group when measured at 28 days (0.68 units/ml,  $P > 0.5$ ). At 28 days, only one subject in group 1 showed a  $\geq 4$ -fold increase in IgG antibody concentration, and the respective geometric mean IgG antibody responses were 6- to 10-fold lower than those of

groups 2 or 3 ( $p < 0.003$  at 7 days; and  $p < 0.01$  at 28 days).

Table 1, below, summarizes the bactericidal antibody responses of the three groups as measured in serum samples obtained at time 0, and 7 and 28 days after the booster vaccination (bactericidal assays were not performed in the sera obtained at 3 days). Prior to the booster vaccination, all five subjects in group 1 had titers less than 1:8 (undetectable), and the majority of adults in groups 2 and 3 also had undetectable bactericidal titers (Table 1). Following vaccination, the geometric mean bactericidal antibody responses at 7 and 28 days paralleled the respective IgG antibody responses. Specifically, at 7 days there was no evidence of an increase in bactericidal antibody titers in group 1, compared to the respective pre-vaccination titers, and the GMT of group 1 at 7 days was 10- to 25-fold lower than the respective GMTs of groups 2 or 3 ( $p < .001$ ). At 28 days, similar respective differences were present ( $P < .006$ ). Further, at 28 days, the proportion of subjects with bactericidal titers  $\geq 1:8$  was only 20% in group 1, vs. 100% for group 2 ( $P = 0.01$ ), vs. 64% for Group 3 ( $P = 0.11$ ).

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Table 1. Complement-Mediated Bactericidal Antibody Responses of Adults to a Meningococcal Polysaccharide Booster Immunization\*

Group	Meningococcal Priming Vaccine	No. Tested	Geometric Mean (Reciprocal Titer) ± 95% Confidence Interval			Percent with Titer ≥ 1:8		
			Pre-	7 Days Post	28 Days Post	Pre-	7 Days Post	28 Days Post
1	Polysaccharide	5	4.0 (4-4)	4.9 (2.8-8.6)	8.5 (1.1-68)	0	20	20
2	Conjugate	18	9.3 (4-21)	136 (69-268)	200 (108-371)	22	100	100
3	Unvaccinated	11	9.2 (3.4-25)	43 (11-174)	88 (17-463)	36	64	73

\*For the booster injection, all subjects were given 1/50th of the usual dose of Menomune (1µg of each polysaccharide, IM).

Statistical Analysis: The group previously given Menomune showed no significant antibody response to the booster. Comparing GMTs of the 3 groups at each time point (Pre,  $p = 0.5$ ; 7 days,  $p < 0.001$ ; and 28 days,  $p = 0.005$ ). Pair-wise comparisons between GMTs of group previously vaccinated with Menomune vs. unvaccinated: 7 days,  $p = 0.01$ ; and 28 days  $p = 0.02$ ). Pair-wise comparisons between GMTs of group previously vaccinated with conjugate vaccine vs. unvaccinated: 7 days,  $p = 0.06$ ; and 28 days,  $p = 0.24$ . Pair-wise comparisons of percent with titers ≥ 1:8 between group previously vaccinated with conjugate vs. unvaccinated: 7 days,  $p < 0.02$ ; and 28 days,  $p < 0.05$  (by Chi square analysis).

The principle finding of this study is that four years after immunization with meningococcal polysaccharide vaccine, healthy adults show much lower anti-meningococcal C serum antibody responses to a booster injection with 1/50th of the usual dose of meningococcal polysaccharide vaccine than adults vaccinated for the first time. In contrast, the magnitude of the booster responses of adults previously vaccinated with an investigational meningococcal conjugate vaccine was similar or higher than that of the adults vaccinated for the first time. These data are consistent with induction of immunologic tolerance to meningococcal C polysaccharide by the prior vaccination with the licensed polysaccharide vaccine, but not by the investigational conjugate vaccine. The conclusion that the initial unconjugated polysaccharide vaccination in adults induced immunologic tolerance is based on the booster responses of 5 subjects, wherein the magnitude of the impairment found was very large (10-fold), and thus unlikely to have resulted from chance alone ( $P \leq 0.01$ ).

One contributing factor in the mechanism for the above-described induction of immunologic tolerance may be the relatively high dose of polysaccharide used in the licensed meningococcal vaccine (50  $\mu\text{g}$ ). That this dose may be excessive is suggested by the excellent immunogenicity of a 1  $\mu\text{g}$  dose in the control adults immunized in the present study for the first time (Figure 1 and Table 1). Also, in a previous study in infants, impaired booster antibody responses to meningococcal C polysaccharide were observed only after vaccination with 25  $\mu\text{g}$  or 100  $\mu\text{g}$  of vaccine, but not after a 10  $\mu\text{g}$  dose (Goldschneider et al. (1973) *J. Infect. Dis.* 128:769-776). In mice, large doses of polysaccharide antigens also have been found to induce immunologic tolerance, whereas lower doses are immunogenic and do not induce a

refractory state to revaccination (reviewed in Halliday, *supra*).

Accordingly, novel methods for boosting anti-meningococcal immune responses in adults, and uses of  
5 first and second meningococcal vaccine compositions in the preparation of medicaments are disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious  
10 variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

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What is claimed is:

1. A method for boosting in an adult subject  
an immune response against meningococcal capsular  
5 antigen, said method comprising:

(a) administering a first vaccine composition  
to said adult subject in order to elicit an immune  
response against a meningococcal species, wherein said  
first vaccine composition comprises a meningococcal  
10 oligosaccharide conjugated to a carrier molecule, and  
further wherein the first composition is administered in  
an amount sufficient to elicit an anti-meningococcal  
immune response, and said immune response is boostable  
upon re-vaccination with a second meningococcal vaccine  
15 composition; and

(b) administering a second vaccine composition  
to said adult subject in order to boost the anti-  
meningococcal response, wherein said second vaccine  
composition is administered to the subject after serum  
20 anti-meningococcal antibody concentrations induced by the  
first vaccine composition have declined to sub-protective  
levels.

2. The method of claim 1, wherein the first  
25 vaccine composition comprises a meningococcal  
oligosaccharide conjugated to a protein carrier.

3. The method of claim 1, wherein the second  
vaccine composition is administered in a suboptimally  
30 immunogenic amount.

4. The method of claim 3, wherein the second  
vaccine composition comprises an unconjugated  
meningococcal polysaccharide.

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5. The method of claim 4, wherein the second vaccine composition is administered at 1/50 of the usual dose.

5           6. The method of claim 1, wherein the second vaccine composition is administered to the adult subject not less than three years after administration of the first vaccine composition.

10           7. The method of claim 1, wherein the first vaccine composition comprises a meningococcal serotype C capsular oligosaccharide immunogen.

            8. Use of a second meningococcal  
15 polysaccharide or oligosaccharide composition in the preparation of a medicament for use in boosting an immune response against a meningococcal species in a subject previously vaccinated with a first anti-meningococcal vaccine composition, wherein:

20           (a) the first composition comprises a meningococcal oligosaccharide conjugated to a carrier molecule, the first composition is administered in an amount sufficient to elicit an anti-meningococcal immune response, and said immune response is boostable upon re-  
25 vaccination with a second meningococcal vaccine composition; and

            (b) the second composition comprises a meningococcal polysaccharide or oligosaccharide immunogen, and is administered to the subject after serum  
30 anti-meningococcal antibody concentrations induced by the first vaccine composition have declined to sub-protective levels.

9. Use according to claim 8, wherein the first vaccine composition comprises a meningococcal oligosaccharide conjugated to a protein carrier.

5 10. Use according to claim 8, wherein the second vaccine composition is administered in a suboptimally immunogenic amount.

10 11. Use according to claim 10, wherein the second vaccine composition comprises an unconjugated meningococcal polysaccharide.

15 12. Use according to claim 11, wherein the second vaccine composition is administered at 1/50 of the usual dose.

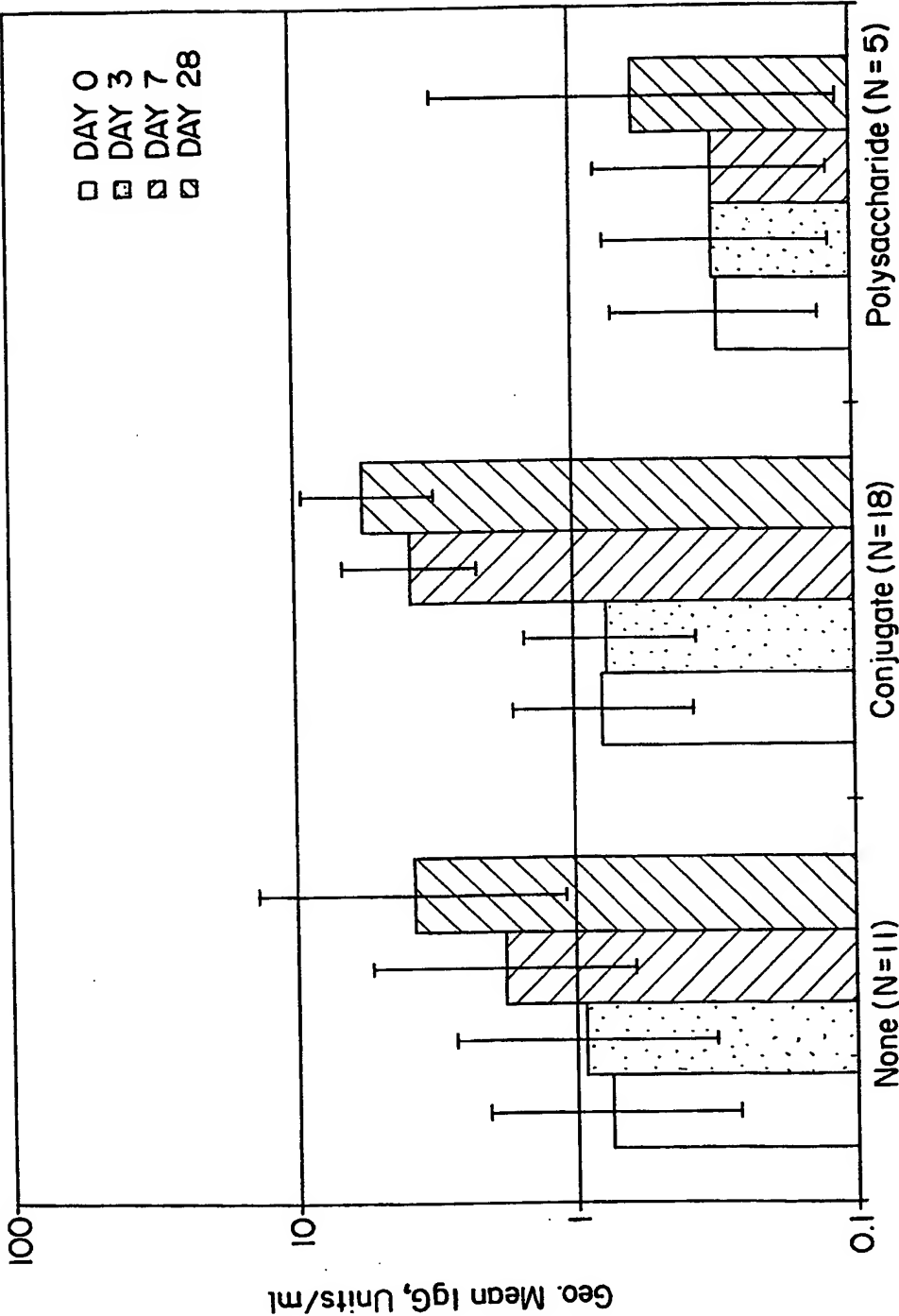
20 13. Use according to claim 8, wherein the second vaccine composition is administered to the adult subject not less than three years after administration of the first vaccine composition.

25 14. Use according to claim 8, wherein the first vaccine composition comprises a meningococcal serotype C capsular oligosaccharide immunogen.

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Previous Priming 4 Yrs. Earlier

FIG. 1

# INTERNATIONAL SEARCH REPORT

Internati Application No  
PCT/US 98/13080

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K39/095 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BRANDT B. ET AL.: "Duration of antibody responses after vaccination with group C <i>Neisseria meningitidis</i> polysaccharide"</p> <p>J. INFECT. DIS, vol. 131, no. sp1, May 1975, pages S69-S72, XP002080206 see page S69, left-hand column, line 1-14 see page S70, right-hand column, line 25 - page S72, left-hand column, line 6</p> <p style="text-align: center;">--- -/--</p>	1-14

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 October 1998

Date of mailing of the international search report

27/10/1998

Name and mailing address of the ISA

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Authorized officer

Covone, M

# INTERNATIONAL SEARCH REPORT

Internati Application No  
PCT/US 98/13080

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KING J. ET AL.: "3 year follow-up and booster response to quadrivalent meningococcal polysaccharide vaccine (QMPV)" PEDIATRIC RESEARCH, vol. 39, no. pt2, 1996, page 106 XP002080207 A622 see the whole document	1-14
A	ANDERSON E.L. ET AL.: "Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults" INFECT. IMMUN., vol. 62, no. 8, August 1994, pages 3391-3395, XP002080208 cited in the application see page 3391, right-hand column, line 24-33 see page 3393, column D, line 18-20 see page 3393, right-hand column, line 36 - page 3394, left-hand column, line 8 see page 3394, left-hand column, line 24-39	1,2,5, 7-9,13, 14
A	WO 94 05325 A (NORTH AMERICAN VACCINE INC) 17 March 1994 see page 4, line 3-7 see example 8	1,2,7-9, 14
P,X	GRANOFF D.M. ET AL.: "Induction of immunologic tolerance in adults by meningococcal C (MenC) polysaccharide (PS) vaccination" CLINICAL INFECTIOUS DISEASES, vol. 25, no. 2, August 1997, page 432 XP002080209 A417 see the whole document	1-14
T	GRANOFF D.M. ET AL.: "Induction of immunologic refractoriness in adults by meningococcal C polysaccharide vaccination" J. INFECT. DIS., vol. 178, September 1998, pages 870-874, XP002080210 see table 1 see page 870, right-hand column, line 24 - page 871, left-hand column, line 33	1-14

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/13080

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1-7 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/13080

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9405325 A	17-03-1994	US 5425946 A	20-06-1995
		AU 4841693 A	29-03-1994
		EP 0658118 A	21-06-1995
		JP 8500607 T	23-01-1996
		NO 950739 A	26-04-1995
		PL 307745 A	12-06-1995